Elucidating the Role of Glyceraldehyde 3-Phosphate Dehydrogenase in Secretion of Extracellular Vesicles in the Cancer Cell System

PhD Synopsis

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1. Background

Extracellular vesicles (EVs) are cell-derived membranous vesicles (from normal or cancerous cells) bearing packages of information within or on their surface. Their content can influence neighbouring or remote cells, and therefore, EVs are considered to play an important role in intercellular communication (Camussi *et al.,* 2010). Different functional molecules (proteins, mRNA, and microRNAs) are transferred between cells with the aid of EVs. The content of EVs is highly variable and dependent of the cell of origin. The EVs in human blood originate from platelets, leukocytes, erythrocytes, endothelial cells, vascular smooth muscle cells, and cancer cells (Nomura, 2017).

EVs are being actively explored to understand their role in promoting repair of cardiac cells post myocardial infarction, formation of pre-metastatic niche, suppression of immune response by tumour derived EVs, and spreading of neurodegenerative diseases (Liu *et al.,* 2022). Therefore, the content of these vesicles is of great significance in understanding complex bidirectional communication occurring under physiological and pathophysiological state (Minciacchi *et al.,* 2017; Ho, J *et al.,* 2022). Moreover, unique ability of EVs to cross blood-brain barriers opens up new opportunities to develop biomarkers for diseases like cancer and neurodegenerative disorders (Daria *et al.,* 2018, Anita *et al.,* 2022). Secretion of EVs by almost all types of cells into biological fluids (blood, saliva, urine, sweat), and their ability to deliver cargos into targeted cells makes them ideal candidates for developing biologically safe vehicles of drug delivery system (Zhen *et al.,* 2022). Taken together, discovery of EVs as communication vehicles have revolutionised our understanding in cell biology and provides

exciting opportunities in developing advanced tools for detecting and treating diseases at early stages of their onset.

In cancer, EVs ensure the cross talk between tumoral cells or between tumoral cells and normal cells, and enable the development of multiple processes, including tumorigenesis, pre-metastatic niche formation, metastasis, and drug resistance. Several immunosuppressive ligands such as programmed cell death ligand-1, FAS ligand and cytotoxic T-lymphocyte antigen-4 have been found on cancer EVs, suppressing immune response towards cancer cells (Annoor *et al.,* 2021). Interestingly, although still under investigation, some studies suggested that cancer cells produce more EVs than normal cells (Vasconcelos *et al.,* 2019; Graves *et al.,* 2004). Tumour-derived EVs have their own specific labels, which distinguish them from the normal cell-derived EVs. Several studies have reported that tumour-derived EVs carry oncogenic membrane proteins or nucleic acids to facilitate tumour progression and a significant difference was observed in the proportion of oncogenic proteins and nucleic acids between normal and malignant cell-derived EVs (Qiao *et al.,* 2019). Tumour cells can communicate with the surrounding tumour and stromal cells via the transport of cargo through tumour-derived EVs; this facilitates tumour invasion, angiogenesis and immune suppression (Blonska *et al.,* 2015). Alternatively, tumour-derived EVs also contribute to the formation of a premetastatic niche for the distant metastasis of cancer (Dong *et al.,* 2021)

The biogenesis of EVs involves their origin in endosomes, and subsequent interactions with other intracellular vesicles and organelles generate the final content of the exosomes. (Kalluri *et al.,* 2020). The role of EVs, as defined by their intracellular endosomal origin remains unclear, as in vivo tools to follow exosomes release and biogenesis are still lacking. The only way to conclusively identify a role for exosomes in cancer is by interfering with their biogenesis, cargo-loading and function. In order to control secretion of EVs and their cargo selection, it is important to have comprehensive understanding of different pathways that

regulate biogenesis of EVs. Recently, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) has been shown to play an important role in EV biogenesis by regulating secretion of EVs via controlling formation of EVs from multivesicular bodies. GAPDH - generally used as internal control in immunoblots and real time PCR- is a glycolytic enzyme that has been recently found to be associated with EVs and with the iron-binding proteins transferrin and lactoferrin on surface of EVs to traffic them into cells from tissue culture medium (Malhotra *et al.,* 2016). It has been observed that GAPDH becomes associated with exosomes during formation of intraluminal vesicles when cells are depleted of iron (Chauhan *et al.,* 2019). Recently, GAPDH has been found to participate in biogenesis of EVs in Drosophila melanogaster. Silencing of GAPDH gene in male testes of drosophila drastically reduced the secretion of intraluminal vesicles into the duct of the gland. Further experiments in Drosophila EV biogenesis model revealed that GAPDH is required for normal generation of ILV in multivesicular bodies and promotes vesicle clustering (G. H. Dar *et al.,* 2021). In this study, we aim to explore the role of GAPDH protein in biogenesis of EVs in human cells. We are also looking to explore the mechanism of GAPDH protein in biogenesis of EVs by developing different in vitro cell models.

2. Review of Literature

Extracellular vesicles (EVs) are cell-released particles ranging in size from 30 to 1000 nm, enclosed within a phospholipid bilayer and which do not replicate. In addition to managing cellular waste, they play a pivotal role in mediating intercellular communication under both physiological and pathological conditions (Thery *et al.,* 2018; Roy *et al.,* 2019; Sousa *et al.,* 2015; Kim *et al.,* 2017). EVs are biocompatible, nano-sized vesicles containing different types of biomolecules, including proteins, RNAs, DNAs, lipids, and metabolites (Fei Teng and Martin Fussenegger; 2020). Their low immunogenicity and ability to functionally modify recipient cells by transferring diverse bioactive constituents make them an excellent candidate for a next-generation drug delivery system. Encapsulation of cargo within EVs enhanced their life time in the blood. Moreover, EVs have the ability to transfer their cargo to recipient cells by crossing biological barriers, such as the blood-brain barrier (Caruso *et al.,* 2019; Doyle *et al.,* 2019; Perut *et al.,* 2019).

EVs vary in size, properties, and biological functions and are mostly classified on the basis of their size and biogenesis pathways. EVs has been mostly classified into exosomes which are 30–140 nm in diameter, microvesicles or microparticles ranging from 100–1000 nm, and apoptotic bodies ranging from 800 to 5000 nm (Cristina *et al.,* 2020). Exosomes are derived from the endolysosomal pathways, which involve inward budding in early endosomes, forming multivesicular bodies (Fei Teng *et al.,* 2020; Figure 1). Compared to other classes, exosomes seem to represent a more heterogeneous population of EVs. On the other hand, micro-vesicles are released via direct shedding from the plasma membrane. Apoptotic bodies, which are less studied, are formed during cytoskeletal rearrangement, being released by outward blebbing of the cell membrane of apoptotic cells (Budnik *et al.,* 2016; Yanez-Mo *et al.,* 2015).

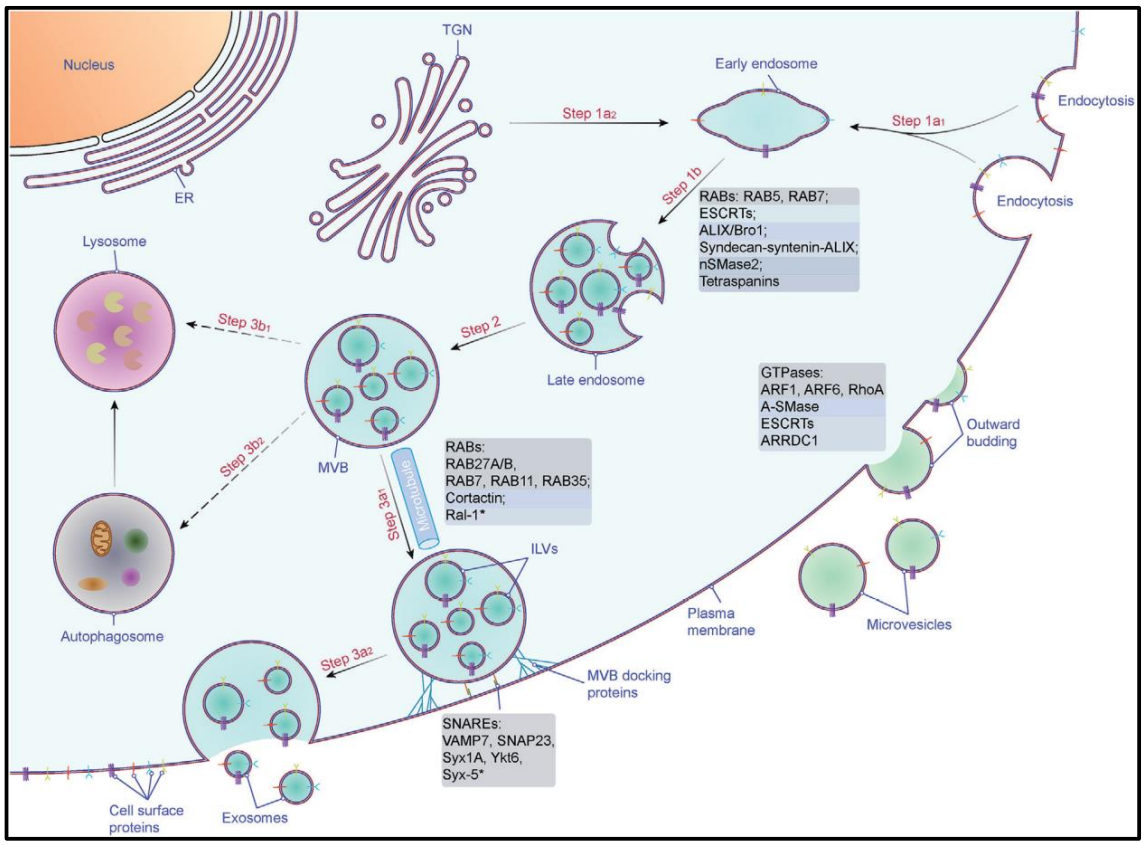

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Figure 1: Biogenesis and secretion of EVs. Two mechanisms of EV biogenesis are illustrated. The process of releasing exosomes into the extracellular milieu contains three distinct steps: exosome biogenesis, intracellular trafficking of MVBs, and fusion of MVBs with the plasma membrane. Early endosomes are formed by the inward budding of the plasma membrane (Step 1a1), or in some cases from the *trans*-Golgi network (TGN) (Step 1a2). Early endosomes mature into late endosomes (Step 1b) and finally generate MVBs, in which process ILVs are formed by inward invagination of the endosome limiting membrane (Step 2). The fate of MVBs can be fusion with the plasma membrane (Step 3a1), which results in the release of exosomes (Step 3a2). Alternatively, MVBs can fuse with lysosomes/autophagosomes for degradation (Step 3b1, 2). Several molecules are involved in the biogenesis (e.g., RABs, ESCRTs, syndecan, ceramide, tetraspanins, etc.), trafficking (e.g., RABs, actin, etc.), and fusion of MVBs with the plasma membrane (e.g., SNAREs). Microvesicles arise from the direct outward budding and fission of the plasma membrane. Several molecules are involved in the biogenesis and release of microvesicles (small GTPases, ESCRTs, ARRDC1, *etc*.).

Abbreviations: EV, extracellular vesicle; ESCRT, endosome sorting complex required for transport; MVB, multivesicular body; ILV, intraluminal vesicle; RAB, RAS-related protein; ALIX, ALG-2 interacting protein X; nSMase2, neutral sphingomyelinase 2; Ral-1, RAL (Ras-related GTPase) homolog; SNARE, soluble NSF attachment protein receptor; VAMP7, vesicle-associated membrane protein 7; SNAP23, synaptosomal-associated protein 23; Syx1A, syntaxin 1A; ARF, ADP ribosylation factor; RohA, Ras homolog family member A; A-SMase, acid sphingomyelinase; ARRDC1, arrestin domain containing protein 1.

EVs were originally reported in 1946 by Chargaff and West as procoagulant platelet-derived particles in normal plasma (Chargaff, E; West, R 1946). Later on, this observation was termed as "platelet dust" by Wolf in 1967 (Wolf, 1967). In 1981, E. G. Trams was the first to observe release of small vesicles of around 40 nm size into cell culture media. He referred these particles as "exosomes" (Trams *et al.,*1981). The first insight about nature of EVs came through a breakthrough finding of Harding and colleagues' famous experiment on transferrin receptor biogenesis where they observed encapsulation of small vesicles into large multivesicular bodies (Harding *et al*., 1984). Till 1990, exosomes were considered as garbage cans used by cells to discard unwanted cellular proteins. Subsequently, a study in 1996 revealed the activation of $CD4^+$ T cells by MHC II enriched exosomes which sparked a renewed interest in exosome biology that led to exponential development of the EV field (Raposo *et al*., 1996).

EVs are generally classified into three major subpopulations by their diameters and biogenesis mechanisms: exosomes (30-140 nm), microvesicles (50-1000 nm), and apoptotic bodies (50-2000 nm) (Willms *et al.,* 2018). Exosomes are generated by inward budding of the endosome membrane to form multivesicular bodies (MVBs) that fuse with the plasma membrane to release mature exosomes *via* a closely regulated process. Conversely, microvesicles and apoptotic bodies are generated by outward budding of the plasma membrane by different processes in viable cells and in cells undergoing programmed death, respectively (Mathivanan *et al.,* 2010). However, all three EV populations can overlap in size, surface markers, and composition to prevent reliable isolation of pure EV samples of specific subtypes by methods in current use. Given this lack of precision, we use the generic term "EV" rather than exosomes or microvesicles. The different terminologies based on function, biogenesis, size or cell of origin, as well as the variety of isolation methods and contexts, and the paucity of validated biomarkers, have led to various misconceptions and even contradictory definitions in the literature (Thery *et al.* 2018). In this context, the International Society for Extracellular

Vesicles (ISEV) has proposed the term "Extracellular Vesicle" as generic nomenclature for cell-released vesicles, and has also recommended minimal requirements for characterisation of EVs while publishing EV related findings (Lotvall *et al.,* 2014). Thus, it was only in 2011 that an important step was taken to unify the nomenclature and the methodologies for isolating EVs. In order to address the issues like absence of consensus in the nomenclature and the limited knowledge on molecular mechanism of EV biogenesis and release, recent guidelines from the International Society for Extracellular Vesicles (ISEV: www.isev.org) were published. These guidelines are important to cover EV separation/isolation, characterization, and functional studies. Moreover, these guidelines advise researchers to refer to EVs by their physical characteristics (size and/or density), biochemical composition or descriptions of the cell of origin, unless their biogenesis pathway is confirmed, for example through live imaging techniques (Thery *et al.,* 2018). EVs are produced by almost all organisms and cell types and allow the intercellular transfer of molecules such as mRNAs, lipids, metabolites, proteins and non-coding RNAs (such as microRNAs and long non-coding RNAs), and even DNA fragments, which can induce phenotypic reprogramming of recipient cells (Fruhbeis *et al.,* 2013; Kosaka *et al.,* 2012; Puhka *et al.,* 2017; Valadi *et al.,* 2007; Vasconcelos *et al.,* 2019; Thakur *et al.,* 2014).

Tumour-derived EVs have been shown to play an important role in inducing tumour phenotypes in recipient cells. Tumour microenvironment (TME) may be altered by EVs, to support tumour growth and survival, induce angiogenesis, tumour cell invasion and metastasis, initiate resistance to cell death, evade immune response, reprogram cellular energy metabolism and drug resistance (Deregibus *et al.,* 2007; Fang *et al.,* 2018; Hoshino *et al.,* 2015; Peinado *et al.,* 2017). EVs undergo several alterations in tumorigenesis, including changes in their biogenesis, release rate and/or protein content, incorporation of oncogenic and mutant macromolecules (Choi *et al.,* 2017). EVs can be recovered from common body fluids, such as

blood and plasma (Caby *et al.,* 2005; Arraud *et al.,* 2014), semen (Aalberts *et al.,* 2012), urine (Gonzales *et al.,* 2010), breast milk (Admyre *et al.,* 2007), nasal secretions (Lasser *et al.,* 2011), saliva (Palanisamy *et al.,* 2010), and even faecal matter (Koga *et al.,* 2011). Since their content reflects their cell of origin, EVs are attractive as potential biomarkers (or source of biomarkers) of certain malignancies, including cancer (Armstrong *et al.,* 2018; Yokoi *et al.,* 2017).

Interestingly, although there is no consensus, some studies show that cancer cells produce more EVs than normal cells (Vasconcelos *et al.,* 2019; Graves *et al.,* 2004). However, others report the lack of statistical difference in the levels of plasma EVs between cancer patients and healthy people (Menck *et al.,* 2017). This conflicting evidence might be attributed to a low inter-laboratory reproducibility and to non-standardization of isolation and purification protocols for EVs (Vasconcelos *et al.,* 2019). Nevertheless, accumulating evidence suggests that tumour-derived EVs pay an important role in all steps of cancer progression, which makes them attractive for developing EV-based liquid biopsies for cancer patients. Compared to conventional biopsies they: (1) are a simpler and less invasive method for early diagnosis, (2) facilitate surveillance of the patient's cancer stage and treatment efficacy, and (3) offer surveillance of patient relapse and prognosis (Hallal *et al.,* 2019; Sedlarikova *et al.,* 2018; Yadav *et al.,* 2018). However, some problems need to be addressed when considering EV-based liquid biopsies in the clinic, such as the time required for analysis, high costs involved, and need to find a compromise between high specificity and high sensitivity (Yadav *et al.,* 2018). Additionally, the EVs retrieved from liquid biopsies are not necessarily representative of the most abundant cell type in a certain tissue (Yuana *et al.,* 2013). Additionally, the EVs' populations, cargo levels, and selective packaging are highly heterogeneous and changeable under different physiological conditions, internal cellular processes or external stimuli (such as temperature and stress), thus offering a mere snapshot of the molecular circumstances in their cell of origin at their point of release from cells (Claridge *et al.,* 2019; Smith *et al.,* 2015). In

addition, the isolation, purification, and characterization of EVs as well as the separation of EV subpopulations, still requires standardization. To end this, many novel methods are under development varying in cost, sensitivity, purity and time required (Nomura, 2017; Doyle *et al.,* 2019; Baek *et al.,* 2016).

EVs are also being considered for cancer therapy, either as cancer vaccines or as drug delivery systems to accumulate certain drugs or siRNAs at specific tumour sites, while reducing toxicity-related adverse effects (Bjorge *et al.,* 2017; Syn *et al.,* 2017; Zhupanyn *et al.,* 2020; Elsharkasy *et al.,* 2020). Given their unique biological and pharmacological characteristics, EVs have attracted interest as mediators of physiological and pathophysiological processes, and have potential as cell-free therapeutics (Iraci *et al.,* 2016). EVs have been reported to play important roles in the regulation of the immune response (Robbins and Morelli, 2014), metastasis of cancer cells (Zhang *et al.,* 2017), propagation of pathogenic proteins involved in neurodegenerative disorders (Varcianna *et al.,* 2019), and are emerging as a source of biomarkers for a variety of diseases (Wiklander *et al.,* 2019). Although heterogeneity of EVs has complicated their molecular characterization, significant advances have been made in our understanding of their biogenesis in recent years (Zhang *et al.,* 2018). Multiple mechanisms of exosome and microvesicle biogenesis have been identified that involve endosomal sorting complexes required for transport (ESCRT)-dependent and ESCRT-independent processes. Multiple cellular components have been identified that participate in these processes including Alix, tumour susceptibility gene 101 protein (TSG101), syndecan-syntenin complexes, the tetraspanin family and lipid rafts (Baietti *et al.,* 2012; Trajkovic *et al.,* 2008). However, a detailed understanding of the regulatory and cargo loading pathways is still under infancy and needs extensive study in order to explore the role of EVs in therapeutics (Worthington *et al.,* 2020). EVs have also attracted considerable interest as potential vehicles for drug delivery, particularly as carriers of macromolecules like non-coding RNAs and

proteins (ELA *et al.,* 2013). Their immunological inertness and ability to cross biological barriers are two important features that can be exploited for therapeutic applications (Johnsen *et al.,* 2014). EVs have been demonstrated to deliver therapeutic small interfering RNAs (siRNAs) and antisense oligonucleotides in multiple therapeutic contexts, including mouse models resulting in suppression of pancreatic cancer and restoration of dystrophin expression for the treatment of, muscular dystrophy, respectively (Kamerkar *et al.,* 2017; Gao *et al.,* 2018; Elsharkasy *et al.,* 2020).

The studies related to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), also known as a 'housekeeping' protein, have revealed multiple functions in cellular processes that are independent of its canonical role in glycolysis. GAPDH is a glycolytic enzyme specifically catalysing the reversible conversion of glyceraldehyde-3-phosphate (G-3-P) to 1,3-diphosphoglycerate (Colell *et al.*, 2009). GAPDH participates in numerous cellular functions, in addition to glycolytic effects. For instance, GAPDH contributes to nuclear tRNA export, DNA replication and repair, endocytosis, exocytosis, cytoskeletal organization, iron metabolism, carcinogenesis, and cell death (Sheokand *et al*., 2014). Although GAPDH is widely used as an internal control, its expression status varies in different human cell lines (Caradec *et al*., 2010). Remarkably increased GAPDH levels are observed in many human cancer types and often correlated with reduced survival (Altenberg *et al*., 2004). GAPDH is also considered as a pro-apoptotic agent (Nakajima *et al*., 2007). Therefore, these deregulations of GAPDH in cancers indicate the inconsistent roles of this enzyme in cell fate determination (Guo *et al*., 2013). GAPDH is regulated by several cancer-related factors. Moreover, PTMs (elaborate PTM), such as phosphorylation, ADP ribosylation, and acetylation, are involved in in cancer cell development, progression, and dissemination (Scarpa *et al*., 2013). As a multifunctional protein, GAPDH also influences cancer cell fate. Thus, GAPDH may be a critical regulator of cancer cell functions and a marker of cancer cell progression and prognosis

(Guo *et al*., 2013). The functional diversity of GAPDH is attributed to its oligomeric nature, post-translational modifications and compartmentalization, allowing it to interact with different proteins (Zhang *et al.,* 2015).

3. Identification of Research Gaps

The secretion of EVs from various types of cells, their existence in almost all kinds of body fluids, and function as mediators of cell-cell communication makes them able to play a crucial role in both physiological and pathological processes. The ability of EVs to carry wide range of molecules including lipids, RNAs, and proteins makes them a promising diagnostic biomarker and therapeutic tool for treatment of cancers and other pathologies. However, an incomplete understanding of EV biogenesis and uptake mechanisms, and a lack of efficient drug loading methods remain critical challenges that need to be addressed (Colombo, 2014; Tang *et al.,* 2019). Current methods of loading therapeutic molecules into EVs such as electroporation, genetic engineering of host cells and chemical conjugation, are limited by low efficiency, toxicity and lack of scalability (Kooijmans *et al.,* 2013; Smyth *et al.,* 2014). Development of methodologies in which the natural properties of EVs are exploited for therapeutic applications is likely to provide a more robust approach to this problem.

The clinical application of EVs is still in its infancy, further investigations will contribute to find out cost and time-effective nanotechnologies for large-scale production of EVs. EVs have considerable potential as bio-delivery vehicles, but our limited understanding of EV biogenesis and loading remains one of the obstacles in developing these natural signalling mediators as a therapeutic tool. EVs have the capacity of carrying specific therapeutic drugs based on the treatment of corresponding diseases, it is necessary to identify appropriate strategies for further tailoring EVs as drug delivery vesicles with high drug loading capacity, high specificity, non-cytotoxic effect and low immunogenicity.

4. Aim

- To understand the role of GAPDH in EV biogenesis in human in vitro cell system.
- To explore therapeutic potential of GAPDH silencing in cancer metastasis.

5. Objectives

- To decipher the role of GAPDH's EV binding domain in biogenesis of EVs by forming multiple mutants of GAPDH protein.
- To evaluate the role of mtGAPDH lacking EV binding activity in secretion of EVs by the cancer cells.
- To explore immunosuppressive activities of EVs via Phosphatidylserine (PS) lipid rafts using GAPDH's EV binding domain that binds via PS lipids to EV surface.

6. Methodology

6.1. Cell culture, isolation and purification of extracellular vesicles.

Human embryonic kidney cells (HEK293T) and HeLa cells will be cultured and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1 X penicillin-streptomycin. Cells will be maintained at 37 °C with 5% CO_2. For isolation of EVs, cells will be seeded in 150 x 20 mm dishes in DMEM + 10% FBS media. Twenty-four hours after seeding, the media of the cells will be replaced with Opti-MEM reduced serum media. Cells will be incubated further for 48 h followed by collection of media in 50 ml falcon tubes. To remove dead and floating cells, the media will be centrifuged at 500 x g for 5 min. The supernatant will be gently transferred into a fresh tube and centrifuged at 3000 x g for 20 min at 4 °C to pellet cell fragments and remaining cell debris. Purified media will be concentrated by tangential ultrafiltration (TFF). Concentrated media will be centrifuged at 10,000 x g for 30

min to remove aggregates and larger particles. Purified EVs eluted from the column will be used for various biological assays.

6.2. Development of different clones of GAPDH mutant gene.

Cloning. The cDNA sequence of human GAPDH will be inserted into the pET28b (+) vector. Six residues of histidine will be inserted at the N-terminus for purification by Ni-NTA chromatography. Standard gene cloning methods such as digestion of vectors and gene of interest by restriction endonucleases followed by ligation of the digested products will be carried out. Insertion of GAPDH gene into vectors will be carried out in DH5α cells using selective antibiotic agarose media plates. Colonies grown in agarose plates will be screened for insertion of GAPDH into the vectors using Colony PCR. Positive colonies will be grown further in LB media for isolation of plasmids using Miniprep plasmid isolation methods. Plasmids will be sent to DNA sequencing labs to confirm the correct sequence of the recombinant vector.

Generation of GAPDH mutants and their interaction with EVs.

Using the human GAPDH cDNA sequence as a template, different types of GAPDH mutants will be developed. The DNA sequence will be inserted into pET-28b (+) for expression in DH5α strains of E. coli cells. The protein will be purified using a Ni-NTA matrix followed by refolding of the protein by gradual dilution of urea concentration. Purified protein will be isolated by gel-filtration column chromatography. For incubation of mtGAPDH with EVs, HEK293T EVs isolated by culturing HEK293T cells in optiMEM media will be incubated with the protein. Excess of unbound protein will be removed by size-exclusion chromatography. After elution, EVs will be characterized by western blotting to examine binding of GAPDH protein on surfaces of EVs.

Western blotting. For western blotting, EV lysates corresponding to 5–10 μg of total protein will be separated on SDS page. EV proteins will be denatured by adding sample loading dye and reducing agent to the EVs followed by incubation of the sample at 70 °C for 10 min. EV lysates would be resolved on SDS-PAGE at 150 V for 1 h in ice-cold Tris-Glycine SDS Running Buffer. Proteins resolved on the gel will be transferred to PVDF Membrane, using a mini transfer blot cell. Primary antibodies diluted in the blocking buffer will be added to the membrane and incubated overnight at 4°C on a shaker. Membranes will be incubated with secondary antibodies for 1 h at room temperature with gentle shaking. Then the membranes will be washed with buffer and developed by chemiluminescence detection. The imaging system will be used to visualize the bands on the membrane.

GAPDH activity assay. A fluorescence-based GAPDH assay will be used to measure enzymatic activity of GAPDH protein of EVs. The assay measures the conversion of NAD+ to NADH in the presence of phosphate and glyceraldehyde-3-phosphate (G-3-P). Under the recommended assay conditions, the rate of NADH production is proportional to the amount of GAPDH enzyme present. A fixed number of EVs will be added to the substrate in a 96-well plate. A fluorescent microplate reader will be used to acquire data using the kinetic mode setting, with λex = 560 nm and λem = 590 nm. Data will be analysed using Data Analysis software.

6.3. Expression of the GAPDH mutant that lacks EV binding activity but retains glycolytic activity in tissue culture cells.

A mutant GAPDH will be engineered which lacks EV binding activity but has glycolytic activity. EVs isolated from HEK293T and HeLa cells would be used to characterize this mutant. This mutant would be expressed in the normal and cancer cell lines after disrupting wild type GAPDH gene by CRISPR/Cas9. This mutant would be cloned into pET28b vector

and expressed in DH5α strains of E. coli cells. After expression, this mutant GAPDH will be purified from DH5α strains of E. coli cells using Ni-NTA matrix. To determine binding of this mutant GAPDH to EVs, western blotting of EVs after incubating them with this mutant would be carried out. Moreover, fluorescent dyes would be conjugated to GAPDH to confirm its binding to EV surface. Expression of this mutant with EVs will further be monitored by GAPDH activity assay.

6.4. Determining immunosuppressive activities of EVs via Phosphatidylserine (PS) lipid rafts using GAPDH's EV binding domain that binds via PS lipids to EV surface.

EVs from cancer and non-cancerous cells would be used to measure T-cell response. EVs would be isolated from cancer and non-cancerous cells as described in objective 1 in order to determine whether PS lipids of cancer EVs have any role in supressing T cell immune response. In vitro T - cell activation assay would be performed after incubating T cells with cancer EVs conjugated with G58 domain of GAPDH, for studying their PS specific immunosuppressive role. G58 peptide conjugated to Flag epitope (G58F) would be expressed in DH5α cells. The protein would be isolated and purified by Ni-NTA chromatography followed by gel filtration. Fixed number of EVs would be incubated with G58F protein. Unbound proteins will be removed by gel filtration. Binding of G58F to EVs would be determined by western blotting, using flag antibody. After characterizing binding of G58F to EVs, specific number of EVs conjugated with G58 would be incubated with T cells. Using T - cell activation assay, the effect of tumour EVs on suppression of T cell activation would be determined. Cells would be stimulated in vitro by adding anti-CD63 and anti-CD28. Using fluorescent labelling, activation of T cells would be studied using flow cytometry in presence of G58F labelled EVs.

7. Expected Outcome

Modulation of GAPDH protein and its pleiotropic effect on EV secretion could be studied. This would lay down a strong platform for future studies. This study will enable us to determine whether GAPDH could be targeted to inhibit secretion of EVs. Moreover, this study may enable the development of GAPDH based-therapies for inhibiting secretion of cancer EVs.

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